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Review

Pot1 and telomere maintenance

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ABSTRACT

Proteins that specifically bind the single-stranded overhang at the ends of telomeres have been identified in a wide range of eukaryotes and play pivotal roles in chromosome end protection and telomere length regulation. Here we summarize recent findings regarding the functions of POT1 proteins in vertebrates and discuss the functional evolution of POT1 proteins following gene duplication in protozoa, plants, nematodes and mice.

1. Introduction

In most eukaryotes, chromosomes end in arrays of GT-rich repeat sequences bound by a set of specific protein complexes [1]. These nucleoprotein structures are commonly referred to as telomeres and they play a crucial role in ensuring genomic integrity by capping the ends of chromosomes. Telomeres also counteract the cumulative loss of terminal DNA sequences inherent to the mechanism of DNA replication by providing a means to restore terminal sequences on linear DNA. Telomeric DNA sequences are added to chromosome ends by a ribonucleoprotein complex containing at its core telomerase reverse transcriptase (TERT) and an RNA subunit (TR), which contains the template sequence for telomere repeat synthesis [2]. At least in vitro, telomerase cannot act on a blunt DNA end, but requires the presence of a single-stranded 3′-extension of the G-rich strand [3]. Such G-strand overhangs are present at chromosome ends and vary in length from 14 nucleotides in ciliated protozoa to 60–100 nucleotides in mammals [4,5].

Proteins that specifically bind G-strand overhangs were first isolated and characterized from the hypotrichous ciliate *Oxytricha nova* [6,7]. The telomere end binding proteins (TEBP) in this and related organisms are comprised of two subunits (α and β) that form a tight ternary complex with telomeric DNA [8]. Structurally, the α subunit is comprised of three oligonucleotide/oligosaccharide binding folds (OB-folds), with a fourth one present in the β subunit [9]. The main ssDNA contacts occur through the two *N*-terminal OB folds of the α subunit, which together with the OB-fold in the β subunit form a deep DNA binding cleft.

Genetic studies in the budding yeast *Saccharomyces cerevisiae* identified Cdc13 as a protein with a critical role in chromosome end protection [10,11]. Like ciliate TEBPs, purified Cdc13 binds tightly to single-stranded G-rich telomeric DNA [11–13] supporting a role in protecting the 3′ overhangs at chromosome ends. Although Cdc13 shares no apparent sequence similarity with the ciliate proteins, the NMR structure of the Cdc13 DNA binding domain revealed the presence of an OB-fold that is structurally similar to the first OB-fold of the TEBP α subunit [14]. Further genetic and biochemical studies showed that Cdc13 plays additional roles in the regulated recruitment of telomerase to chromosome ends and in coordinating the synthesis of G-rich and C-rich strands [11,15–18].

Examination of the fission yeast genome database led to identification of an uncharacterized open reading frame that displayed weak sequence similarity to the amino-terminal OB-fold of *Oxytricha* TEBP α [19]. Deletion of the corresponding genomic region triggered rapid loss of telomeric and subtelomeric DNA sequences followed by chromosome end fusions and segregation defects. The protein was named Pot1, for protection of telomeres, in recognition of these phenotypes. Although most cells lacking

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Pot1 die as a consequence of chromosomal instability, random fusion of chromosome ends results in the circularization of all three chromosomes in a subset of cells. Perhaps surprisingly, fission yeast cells with circular chromosomes multiply readily and form survivor strains [19]. A similar phenotype has also been observed in fission yeast strains deleted for other factors required for telomere maintenance such as the checkpoint kinases Rad3(ATM) and Tel1(ATR) [20] or telomerase components [21–24]. Consistent with a role in binding to the 3′-overhang at chromosome ends in vivo, fission yeast Pot1 binds to the G-rich strand of telomeric DNA in vitro but not the C-rich strand or double-stranded DNA [19,25].

Further genetic studies, together with analysis of Pot1 truncation and point mutants, indicated that fission yeast Pot1 functions in telomere length regulation in addition to its role in protecting chromosome ends from degradation and fusion [26,27]. Comparative sequence analysis revealed the presence of Pot1-like open reading frames in most eukaryotic genomes including many other fungi, plants, nematodes and vertebrates [28–33]. In all species where Pot1 homologs have been examined experimentally, the proteins function in telomere maintenance, but the precise roles played in end protection and telomere length regulation seem to vary considerably between organisms.

2. Vertebrate POT1 functions

2.1. Checkpoint prevention

The dramatic effect of *pot1*$^+$ disruption in *Schizosaccharomyces pombe* led to the expectation that removal of vertebrate POT1 would also cause rapid telomere loss and chromosome end fusions. However, knockdown of human POT1 was found to mainly cause telomere elongation instead of degradation, and telomere fusions were quite rare [34–37]. Other effects of the knockdown varied with cell type but they included decreased proliferation, genome instability and/or a mild DNA damage response triggered at telomeres. These findings suggested that human POT1 might function predominantly in telomere length regulation rather than in the protection of telomeres, or more specifically the G-overhang. A more subtle role for hPOT1 in telomere protection was uncovered after it was realized that the C-rich strand ends in the sequence CCAATC-3′ rather than in other permutations of the telomeric repeat [38]. As POT1 knockdown caused this endogenous 5′ terminal sequence to become randomized, POT1 either protects the 5′ end or regulates processing of the C-strand [37].

As knockdown experiments only reduced the level of POT1 but did not completely eliminate it from cells, the full extent of a POT1 loss phenotype remained unclear until the chicken and mouse *POT1* genes were disrupted. In both cases, POT1 was found to be essential because it prevents the telomere from activating a catastrophic DNA damage response [39–41]. In chicken cells, POT1 depletion results in an acute DNA damage response at telomeres, G2 arrest, increased G-overhangs and rapid telomere growth [41]. Disruption of the two *POT1* genes in mouse embryonic fibroblasts causes reduced proliferation, a severe telomeric DNA damage response (TIFs or telomere dysfunction-induced foci), chromosome reduplication, increased sister telomere recombination and resection of the telomeric C-strand to give long G-overhangs [39,40]. The chromosome reduplication occurs as a result of a strong block to cell division, which eventually leads to DNA re-replication without the cells first undergoing mitosis [42]. Telomere loss is uncommon in POT1-deficient chicken and mouse cells, and telomere fusions are only modestly increased compared to control cells with normal levels of POT1. The continued presence of the G-strand overhang in the absence of POT1 explains the lack of widespread chromosome end fusions as the single-stranded overhang prevents repair by the non-homologous end-joining pathway [43]. The relative stability of G-overhangs without POT1 also suggested that other proteins can substitute for POT1 by binding to the overhang and protecting it from degradation.

Further examination of the DNA damage response caused by POT1 removal revealed that it is mediated by ATR rather than ATM [44–46]. This contrasts with the effect of TRF2 removal which results in ATM activation [45]. The ATR damage response indicated that the factor replacing POT1 on the overhang was most likely Replication Protein A (RPA) because ATR recruitment depends on an interaction between its binding partner ATRIP and RPA-coated ssDNA [47,48]. RPA also helps to recruit another complex involved in ATR activation, the Rad9/Rad1/Hus1 checkpoint clamp. ChIP experiments have since confirmed that RPA is loaded onto the telomere after POT1 removal in chicken cells (Churikov and Price, unpublished results). The 3′ terminal stretch of single-stranded DNA on a telomere is an ideal substrate for ATR activation because loading of the checkpoint clamp occurs preferentially at a 5′ double-stranded/single-stranded DNA junction (Fig. 1) [47]. Given that the 3′ overhang is essential for telomerase action, it appears that the evolution of a unique telomeric G-strand DNA binding protein was essential in order to actively exclude RPA and prevent checkpoint activation at chromosome ends.

RPA functions in a wide array of processes necessary for DNA replication and repair. Consequently, it is far more abundant in cells than POT1. This means that for POT1 to effectively compete for single-stranded DNA binding it must either bind telomeric G-strand DNA with a higher affinity than RPA or be actively and specifically recruited to the telomere. In fact both scenarios appear to be true. POT1 has a binding partner TPP1 (ACD), which is responsible for POT1 import into the nucleus [49,50]. Once in the nucleus, the POT1-TPP1 heterodimer becomes associated with the telomere through binding of TPP1 to TIN2, a protein that in turn interacts with TRF1 and TRF2, which directly bind double-stranded telomeric DNA [51,52]. These interactions provide a

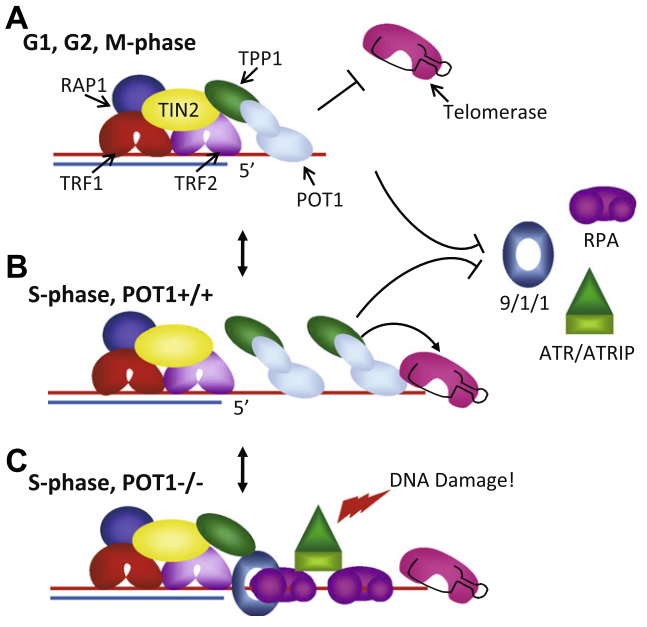

Fig. 1. POT1 regulation of telomerase and RPA access to the 3′ overhang. (A) POT1 prevents telomerase access by sequestering the DNA terminus. Telomere is shown in a closed state. (B) During S-phase an alternative mode of binding makes the 3′ terminus accessible to telomerase. TPP1 stimulates telomerase processivity. (C) POT1 removal leads to RPA binding and activation of an ATR-mediated DNA damage response.

means to raise the local concentration of POT1 at the telomere and hence may help POT1 compete with RPA for overhang binding. In chicken cells, the interaction with TPP1 is not required if the POT1 DNA-binding domain is overexpressed with a nuclear localization sequence (Wei and Price, unpublished results).

The POT1-TPP1 heterodimer also has a higher intrinsic affinity for telomeric DNA than does RPA. In physiological salt, human POT1 and RPA bind 35 nt telomeric G-strand DNA substrates (the optimal binding site size for RPA) with similar affinity (Churikov and Price, unpublished results) [86]. However, association of TPP1 with POT1 increases the binding constant for telomeric DNA by 5–10-fold [53,54]. TPP1 association also augments POT1's ability to discriminate against RNA binding, thus reducing spurious binding to the telomeric RNA sequences found in TERRA and other RNAs [55]. Thus, the binding of TPP1 to POT1 enhances the ability of POT1 to compete with RPA via multiple routes.

2.2. Telomerase and telomere length regulation

A number of studies have suggested that single-stranded telomeric DNA binding proteins inhibit telomerase activity. Biochemical experiments with TEBPs from ciliated protozoa were first in showing that Pot1-like proteins could sequester a DNA terminus and render it inaccessible to telomerase [3]. The mechanistic basis for the inhibition of telomerase was nicely illustrated by the crystal structure of the ternary complex of TEBP α and β with telomeric DNA [56]. In this structure, the 3′ terminus of telomeric DNA is deeply embedded in a cleft between the α and β subunits, rendering it inaccessible to sequence addition by telomerase and to nucleolytic degradation.

Initial in vitro experiments with fission yeast and human POT1 indicated that POT1 also inhibits extension of a telomeric primer by telomerase and further supported a capping function [25,57]. Consistent with these biochemical studies, reducing the level of POT1 in human cells by RNA interference resulted in telomere elongation [34,58] as did the knockout of chicken POT1 [46].

A primary role for POT1 in inhibiting access of telomerase to the telomere was at first difficult to reconcile with the observation that overexpression of human POT1 also results in telomere elongation [59]. An explanation for why reducing or increasing the amount of POT1 both result in telomere elongation may lie in the ability of TPP1 to interact with both POT1 and TIN2 [60]. The TIN2–TPP1 interaction links POT1 to TRF1, TRF2 and RAP1 so a bridge can be formed between the double and single-stranded part of the telomere. This bridge can force the telomere into a closed conformation in which the 3′ end is inaccessible to telomerase [61]. At shorter telomeres, this bridging complex (shelterin) is less stable and the telomere will be more likely to adopt an open and linear conformation accessible to telomerase. By this mechanism, telomere length information can be transmitted to the 3′ end where it limits extension of longer telomeres but permits telomerase action at short telomeres [62]. POT1 overexpression can disrupt the closed conformation when different POT1 molecules bind to TPP1-TIN2 and the G-strand overhang thereby breaking the protein bridge. Such a mechanism implies that a closed conformation of the telomere rather than simple POT1 binding to single-stranded telomeric DNA is required to inhibit telomerase in vivo.

The importance of DNA sequestration versus telomere conformation in regulating telomerase access is illustrated by an analysis of telomere growth in POT1 conditional cells [46]. If ATR signaling is inhibited, the cells no longer arrest in late S/G2 after POT1 removal but continue to cycle. The ATR inhibition also results in a much reduced rate of telomere elongation. This finding indicates that the rapid telomere growth in arrested cells stems both from the lack of POT1 binding to the overhangs and from keeping the telomeres in an open conformation.

Indeed, more detailed biochemical analyses of the effect of Pot1 on telomerase activity revealed a critical role for the telomeric sequence permutation present at the 3′ end of the primer DNA [63]. Whereas hPOT1 inhibited telomerase activity on some DNA substrates, it actually stimulated the extension of other telomeric oligonucleotides. The crystal structure of hPOT1 in complex with telomeric DNA illustrated that the *N*-terminal OB-fold binds to the sequence TTAGGG, while an adjacent OB-fold interacts with four additional nucleotides [64]. The terminal G on a substrate primer ending in TTAG is thus buried in a pocket within the second OB-fold and is inaccessible to telomerase. If POT1 instead binds in a register that leaves at least eight nucleotides free at the 3′ end, the substrate is extended by telomerase with increased activity and processivity [63]. Stimulation of telomerase on such a primer is at least in part mediated by POT1 inhibiting the formation of G-quadruplex structures [65].

Subsequent in vitro studies with the human POT1–TPP1 complex revealed additional complexity in the effect of POT1 on telomerase activity. Not only is the position of the POT1 binding site relative to the 3′ end critical for inhibition or modest stimulation of telomerase, but the presence of TPP1 was found to further stimulate telomerase activity [53,54]. The POT1–TPP1 complex increases the processivity of telomerase by slowing primer dissociation and facilitating telomerase translocation [66]. Interestingly, POT1–DNA interactions are insufficient to account for telomerase stimulation by the POT1–TPP1 complex; instead the effect requires specific contacts between TPP1 and telomerase [67]. Mix-and-match experiments between telomerases and telomeric proteins from different vertebrates indicate that highly species-specific protein-protein and/or protein–RNA contacts are required for stimulation of telomerase activity and possibly also for the recruitment of telomerase to the telomere [67,68].

2.3. Yin and Yang

The picture that emerges from these studies suggests a role for POT1 as the gate keeper of the telomeric 3′ end (Fig. 1). On one hand, POT1 binding to the single-stranded region of the telomere prevents binding of RPA, which could result in checkpoint activation and downstream repair events including degradation, recombination, and ligation of chromosome ends. As part of the shelterin complex POT1 also restricts access of telomerase to chromosome ends. However, the stimulation of telomerase by POT1-TPP1 in vitro suggests that these two proteins have a more complex role in regulating telomere length. Instead of telomere elongation requiring dissociation of POT1 from the G-strand overhang, the shelterin complex is likely to be disrupted in a manner that leaves POT1-TPP1 associated with the chromosome end where it then stimulates telomerase activity. How this transition is mediated is presently unclear, but the involvement of several kinases in telomere length regulation in yeast suggests that post-translational modifications of telomeric proteins are likely to be involved [20,69–71].

3. Potl evolution: broader roles in telomere biology

Although telomere proteins generally exhibit a rapid rate of evolution as compared to other DNA-binding factors, members of the Pot1 and TRF protein families are somewhat unique in that their genes commonly undergo duplication events followed by functional divergence of the resulting orthologs [72]. In the case of Pot1, some orthologs have closely related functions as exemplified by POT1a and POT1b from mouse [39,73]. However, the orthologs from certain ciliates, plants and *Caenorhabiditis elegans* have evolved very different, and sometimes quite unconventional, roles in telomere metabolism.

Mouse POT1a and POT1b are closely related (72% sequence identity), they both bind TPP1, and they function together at telomeres to perform the same general role as the single Pot1 protein from humans, chickens or *S. pombe* [74]. As discussed above, double *Pot1a/b* gene disruption results in a DNA damage response, resection of the C-strand to give long 3′ overhangs and elevated telomere recombination [39,40]. Comparison of single and double POT1 knockout cell lines has revealed that the two proteins have only partially overlapping functions [39,75]. Either protein can prevent telomere recombination, however POT1a is primarily responsible for preventing ATR activation, while POT1b prevents C-strand resection. Interestingly, POT1a and POT1b exhibit similar in vitro binding affinity for G-strand DNA, so it is currently unclear why POT1a is better able to compete with RPA in vivo to prevent ATR signaling [49]. The ability of POT1b to regulate C-strand processing to give 3′ overhangs of the correct length and 5′ terminal sequence is specified by a domain that is not shared by POT1a [49].

POT1 genes have also undergone gene duplication in some ciliates but in this case the encoded proteins have diverged further (35–45% identity) and they can have quite different functions. In *Euplotes*, one Pot1 ortholog, the telomere end-binding protein (TEBP), probably plays a canonical role in telomere protection as it is present at the telomere throughout the cell cycle [76,77]. However a second Pot1 ortholog, the replication telomere protein (RTP), is present only in *S*-phase cells where it localizes specifically to the sites of DNA replication, suggesting a specialized role in telomere replication [78]. *Tetrahymena* also has two Pot1 proteins, Pot1a and Pot1b. Pot1a is present at macronuclear telomeres in vegetative cells where it functions in a similar manner to human, chicken and *S. pombe* Pot1 as it prevents checkpoint activation and participates in telomere length regulation [79]. When Pot1a is depleted, *Tetrahymena* cells undergo a rapid cell cycle arrest and telomere growth, however G-overhang structure remains largely unchanged. Pot1b is only 42% identical to Pot1a and it appears to have a very different function. Pot1b is not expressed in vegetative cells and thus is not required for normal cell growth or telomere maintenance (Heyse et al., in preparation). Instead, it is found in the developing macronucleus of mated cells and the timing of expression corresponds to the period when the chromosomes are being fragmented and new telomeres synthesized. Pot1b is present on chromosomes in the region where the new telomere will be generated suggesting that the protein has evolved a role in new telomere addition.

Plant *POT1* genes are particularly interesting because comparison of *POT1* family members from various land plants has revealed multiple evolutionary events resulting in sporadic gene duplication that may or may not be accompanied by functional divergence. Plant *POT1* genes were first identified in the genome of *Arabidopsis thaliana* [80,81]. Two of the genes, *POT1a* and *POT1b* encode proteins with domains that are homologous to the two OB-folds of human POT1. The third gene, *POT1c* contains only the first OB-fold domain and appears to have arisen via a recent duplication of the N-terminal region of *POT1a* [81]. While the function of Pot1c remains unclear, both POT1a and POT1b have turned out to be components of telomerase rather than telomere capping components [28,81,82]. The first clue that POT1a might not function in telomere capping came from the discovery that plants null for POT1a undergo gradual telomere erosion, a phenotype analogous to that seen in a TERT mutant [80]. POT1a was then shown to associate with telomerase and to be present at telomeres predominantly in *S*-phase [82]. These observations raised the possibility that POT1a might be a telomerase recruitment factor.

Subsequent experiments have shown that neither POT1a nor POT1b can bind to telomeric DNA, instead both proteins interact with telomerase RNA [32]. *A. thaliana* has two different telomerase RNA molecules, TER1 and TER2, that are each capable of interacting with TERT to form catalytically active enzyme. POT1a binds to TER1 while POT1b binds to TER2 [83]. Given that POT1a depletion causes both telomere erosion and a decrease in telomerase activity, the POT1a-containing telomerase complex appears to positively regulate telomerase activity. However, POT1b depletion causes an increase in telomerase activity, suggesting that this complex may negatively regulate telomerase. How the two opposing activities function to regulate in vivo telomerase activity is unclear. It is striking that POT1a and POT1b null cells lack the characteristic hall marks of telomere uncapping, e.g. rapid telomere loss, C-strand resection and telomere fusions. Thus, in *A. thaliana* chromosome end-protection appears to be achieved by the newly identified CST (Ctc1, Stn1, Ten1) complex rather than by POT1a or POT1b [84].

Although whole genome duplication is a common event in plants, examination of *POT1* genes from a wide variety of plant lineages has revealed that *POT1* gene duplication is comparatively rare suggesting that it is selected against. Out of ~14 land plants examined to date, *POT1* gene duplications have been found only in maize (a monocot), cauliflower and *Arabidopsis* sp. (dicots and members of the Brassicaceae family) [32,33]. Surprisingly, evolution of POT1 function from a telomere capping protein into a telomerase component seems to be equally sporadic. Analysis of DNA binding capacity indicates that while the POT1 proteins from many plants are unable to bind telomeric DNA, those from certain dicots (maize and asparagus), the green algae *Ostreococcus lucimarinus* and the moss *Physcomitrella patens* recognize the telomeric G-strand sequence [32,85]. Moreover, disruption of the single *POT1* gene from *Physcomitrella* results in loss of telomeric DNA, elongated G-strand overhangs and rampant telomere fusions. These phenotypes are characteristic of telomere uncapping rather than loss of telomerase function indicating that *Physcomitrella* Pot1 plays a role in telomere protection rather than telomerase recruitment.

Analysis of Pot1-related proteins from *C. elegans* has revealed yet more unexpected aspects of Pot1 evolution. *C. elegans* contains no less than four genes that share sequence homology with the OB-fold encoding regions of vertebrate Pot1 genes [30,31]. However, each gene shares homology with only one of the two vertebrate OB-fold domains that make up the DNA binding domain. *C. elegans* chromosomes are unusual in that some telomeres have a 3′ overhang while others have a 5′ overhang [31]. Two of the Pot1-related proteins, CeOB1 and CeOB2, are present at telomeres and seem to be tailored to bind these 3′ or 5′ overhangs. CeOBl, which shares more similarity with the second OB-fold of vertebrate Pot1, binds preferentially to the TTAGGGC-3′ sequence found at 3′ overhangs. In contrast, CeOB2 is more similar to the first OB-fold of vertebrate Pot1 and binds preferentially to the AATCCCG-5′ sequence found at 5′ overhangs. The phenotypes caused by CeOB1 and CeOB2 loss are slightly different. Loss of either protein causes telomere elongation and an increase in telomere recombination products. However, the telomeres of CeOB1 mutants become somewhat longer and less heterogeneous than those of CeOB2 mutants. It is noteworthy that *C. elegans* telomeres exhibit less strand-specific sequence bias (GCCTAA versus GGCTTA) than that found in other organisms. Thus, one wonders whether the generation of both 3′ and 5′ overhangs and the evolution of CeOB1 and CeOB2 to bind one or the other overhang reflect this diminished sequence bias.

One of the four Pot1-like genes in *C. elegans*, *MRT1*, was identified through a genetic screen for mutants that compromise telomerase activity [30]. *MRT1* encodes a multifunctional protein with an *N*-terminal domain that is homologous to the second OB-fold of Pot1 and a *C*-terminal domain that is homologous to the nuclease domain of the SNM1 nuclease family. The encoded protein displays hallmarks of both Pot1 and SNM1 nucleases as it binds to both 3′ and 5′ telomeric overhang sequences and it displays 3′–5′

exonuclease activity. While the precise function of MRT1 is unknown, it is required for telomerase function, as MRT1 deficient cells display progressive telomere shortening.

The involvement of Pot1-like proteins in telomere maintenance in a wide variety of eukaryotes suggests that this factor was recruited early to chromosome ends to participate in their protection and maintenance. The duplication and evolution of distinct functions in many species today illustrates the extreme fluidity of telomere and telomerase associated proteins and their ability to adapt to changing environments and challenges.

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